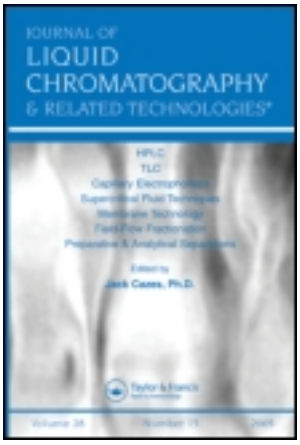

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ljlc20

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Accepted author version posted online: 23 Oct 2012.Published online: 09 Jun 2013.

Journal of Liquid Chromatography & Related Technologies, 36:2107–2124, 2013

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826076.2012.712934

NOVEL REAGENT FOR THE SENSITIVE DETERMINATION OF FREE FATTY ACIDS BY HPLC WITH FLUORESCENCE DETECTION AND IDENTIFICATION WITH MASS SPECTROMETRY AND APPLICATION TO SEVERAL MEDICINAL HERBS

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□ *A new fluorescence reagent 2-(2-(4-(dimethylamino)-phenyl)-1H-phenanthro-[9,10-d]-imidazol-1-yl)-ethyl-4-methylbenzenesulfonate (PPMB) has been designed for fatty acids labeling. Fifteen fatty acids containing six saturated and nine unsaturated fatty acids were used to evaluate the analytical potential of this reagent. The derivatization reaction of PPMB with fatty acids was carried out in the presence of K_2CO_3 catalyst at 95° C for 40 min in* N, N-*dimethylformamide solvent. The separation of fatty acids was achieved on a reversed-phase Hypersil BDS C_8 column (4.6 × 200 mm, 5 μm) with a good baseline resolution. The fluorescence excitation and emission wavelengths were set at λ_{ex} 260 nm and λ_{em} 430 nm, respectively. The identification was carried out by the online APCI-MS in positive-ion detection mode. Results showed the fatty acid derivatives not only enabled sensitive fluorescence detection but also had high MS ionizability. Excellent linear responses were observed using fluorescent detection with coefficients of >0.9993. Detection limits, at a signal-to-noise ratio of 3:1, were in the range of 29.88–69.37 fmol. Fatty acid extracts from* Lycium chinese mill, Long pepper, *and* Evodiae fructus *were successfully analyzed with satisfactory results.*

Keywords derivatization, *Evodiae fructus*, fatty acids, HPLC, *Long pepper, Lycium chinese mill*

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INTRODUCTION

Fatty acids are widely distributed in nature and they are the constituents of all plant cells, where they act as membrane components, storage products, metabolites, and as a source of energy.[1] They also play significant roles in immune system regulation and diverse physiological processes affecting normal health and chronic diseases,[2,3] such as the regulation of plasma lipid levels, cardiovascular and immune function, insulin action, neuronal development, and visual function.[4] Therefore, the determination of fatty acid composition is equally important. However, most fatty acids show neither natural UV absorption nor fluorescence. Hence, the accurate detection of them using absorptiometry is fairly difficult. The method commonly used for analysis is detection of fatty acid derivatives by methyl esterification with GC or GC/MS.[5,6] In recent years, the application of HPLC for analyzing fatty acid derivatives has been widely adopted. In contrast with GC, it allows the fatty acids to be converted to a large number of different derivatives. Several HPLC methods have been developed by employing precolumn derivatization techniques for the analysis of saturated and unsaturated fatty acids.[7] Therefore, a large number of fluorescence labeling reagents which have a fluorophore group and a reactive functional group for carboxylic acids have been developed and applied to the trace analysis of fatty acids in biological samples. Among these, the bromoalkyl reagents (Br-MMC, Br-MDC, Br-MAC, etc.),[7–10] diazomethane reagents (ADAM, PDAM, etc.),[11,12] amine reagents (NEDA, 9-AP, ADC, etc.),[13–15] alcohol reagents (HMA, HEC, etc.),[16,17] and sulfonate reagents (NOEPES, NE-OTf, AE-Otf, etc.)[18–20] are commonly used. However, most of them show some shortcomings, such as short detection wavelengths, poor stability, low detection sensitivity, and serious interferences for the biological sample analysis;[21] therefore, development of a highly sensitive and stable fluorescence reagent for the determination of fatty acids is of great importance.

In this study, 2-(2-(4-(dimethylamino)-phenyl)-1*H*-phenanthro[9,10-d]imidazol-1-yl)ethyl-4-benzenesulfonate (PPMB) has been developed as a new labeling reagent for the determination of saturated and unsaturated fatty acids. Results indicated that PPMB can react with fatty acids in DMF solvent to provide high yields of esters in the presence of K_2CO_3 catalyst. The optimal derivatization conditions were investigated. The detection and identification of fatty acid derivatives were carried out by fluorescence and APCI-MS in positive-ion detection mode. Meanwhile, the fluorescence detection responses were compared with those obtained by using 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene (TSPP)[22] as labeling reagent previously reported in our laboratory. Linearity, detection limits, and precision of the procedure were also evaluated and compared with the reported data.

Lycium chinese mill, Long pepper (*Piper longum*), and *Evodiae fructus* are famous medicinal herbs in China, and well-known for their dietary properties and medicinal values.[23–25] Many bioactive substances have been determined in these herbs, such as phenolic acids and flavonoids in *Lycium chinese mill*,[26] twelve alkaloids in crude *Evodiae fructus*,[27] and four acidamides in *Piper longum*.[28] To the best of our knowledge, there have been no available literature data about fatty acid composition in these herbs. Thus, accurate analysis of these compounds is not only important for better clarifying the activity of medicinal herbs, but also imperative to the quality control. The other objective of this work was to employ the proposed method to simultaneously determine fifteen fatty acids in these herbs.

EXPERIMENTAL

Instrumentation

The HPLC system was an Agilent HP1100 HPLC-MS series (Waldbronn, Germany) and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), and a fluorescence detector (FLD) (model G1321A). Mass spectrometer 1100 Series LC/MSD Trap-SL (ion trap) from Bruker Daltonnik (Bermen, Germany) was equipped with an atmospheric pressure chemical ionization (APCI) source (in positive ion mode). The semi-preparative HPLC system, used to obtain the single PPMB-C19 and TSPP-C19 derivatives, was Waters Delta 600 (Waters, Japan) and consisted of an online degasser (AF), a Water 600 controller with Waters 2489 UV/visible detector, and an auto-fraction collector III.

Chemicals and Materials

Standards of saturated and unsaturated fatty acids were purchased from Sigma Reagent Co. (USA). Spectroscopically pure acetonitrile was purchased from Germany (Merck, KGAa). *N,N*-dimethylformamide (DMF) was purchased from Tianjin Chemical Reagent Co (Hebei, Tianjin, China). Potassium carbonate (K_2CO_3), pyridine, and chloroform were of analytical grade obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). DMF was redistilled prior to use. *Lycium chinese mill, Long pepper,* and *Evodiae fructus* which had been dried, were provided by the Northwest Plateau Institute of Biology, Chinese Academy of Sciences. 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimi-dazole-[4,5-f]-9,10-phenanthrene (TSPP) was prepared according to the method previously described in our laboratory.[22]

Synthesis of Labeling Reagent 2-(2-(4-(dimethylamino)phenyl)-1H-phenanthro[9,10-d]-imidazol-1-yl)-ethyl-4-methylbenzene-sulfonate (PPMB) Synthesis of N,N-dimethyl-4-(1H-phenanthro [9,10-d]imidazol-2-yl)aniline(DPIA)

N,N-Dimethyl-4-(1*H*-phenanthro[9,10-d]imidazol-2-yl)aniline (DPIA) was synthesized according to the method as follows: 9,10-Phenanthraquinone (10 g), 4-(dimethylamino)benzaldehyde (10 g), and ammonium acetate (74 g) were fully added in a 500-mL of round-bottom flask, which were mixed with glacial acetic acid (300 mL). The contents of the flask were heated at 80–90°C for a period of 3 hr with vigorous stirring. After cooling, the mixture was transferred into 600 mL of water, and pH of the solution was adjusted to 7–8 with ammonium hydroxide. The precipitation was recovered by filtration, washed with water, and dried for 48 hr at room temperature. The crude compound was recrystallized twice from a mixed solvent (acetonitrile/DMF, 5:1, *v*/*v*) to afford yellow crystal, yield 94%, m.p. > 280°C. Found, C 81.82, H 5.63, N 12.37; Calculated, C 81.87, H 5.68, N 12.45; IR (KBr): 1652.68 (phenyl– N–H), 1611.53 (phenyl–C=N–); 1490.68, 1530.08 (Ph); 1200.13 (C-N) 1459.90, 1439.54, 1362.33 (C–H), 808.83, 754.94, 736.15, 724.59, 717.50. APCI-MS: *m*/*z*: 338.1 $[M+H]^+$.

Synthesis of 2-(2-(4-(dimethylamino)phenyl)-1H-phenanthro[9,10-d]imidazol-1-yl)ethanol (DPIE)

DPIA (10 g), KOH (0.2 g), and 1,3-dioxolan-2-one (8.0 g) in DMF (100 mL) were rapidly heated to reflux for 3 hr with vigorous stirring. After cooling, the contents were transferred into 300 mL of water. The precipitation was removed by filtration, washed successively with water and 60% ethanol solution (ethanol/water 3:2, *v*/*v*). The crude compound was dried at room temperature for 48 hr and then recrystallized twice from acetonitrile/DMF mixed solvent (5:1, *v*/*v*) to afford white acicular type crystals, yield 78%, m.p. 239.4–240.8°C. Found, C 78.65, H 6.04, N 10.98; Calculated, C 78.71, H 6.08, N 11.02; IR (KBr), 3178.24 (−OH); 1620(phenyl–C=N–); 1552.20, 1519.20, 1488.98 (pH); 1447.28, 1392.94, 1362.93 (C–H); 1065.73 (C–O), 818.87, 770.9, 750.60, 741.23, 721.02. APCI-MS: *m*/*z*: 382.0 $[M+H]^+$.

Synthesis of PPMB

To a solution containing 5.0 g solid DPIE and 15 mL pyridine (0°C) in a 200-mL round-bottom flask, 7.5 g *p*-toluenesulfonyl chloride was added within 30 min with vigorous stirring. After the solution was kept at 0°C

for 6 hr with stirring, the contents were kept at room temperature for another 4 h with vigorous stirring, and then the solution was transferred into 100 mL of ice water with vigorous stirring for 30 min. The precipitation was recovered by filtration, washed with the distilled water and dried at room temperature for 48 hr. The crude product was recrystallized twice from acetonitrile to afford 6.3 g of white crystals, yield 90%, m.p. 131.8–132.3°C. Found, C 71.71, H 5.42, N 7.87, S 5.98; Calculated, C 71.75, H 5.46, N 7.84, S 5.99. IR (KBr), 1611.98 (phenyl–C=N–); 1547.08, 1515.93, 1485.63, (phenyl); 1444.70, 1424.18 (C–H); 1355.01 (–C–SO_2^-); 1198.31, 1174.91 (phenyl–S–), 821.63, 809.76, 764.70, 664.54. *m/z*: 536.0 $[M+H]^+$.

LC and MS Conditions

HPLC separation of fatty acid derivatives was carried out on a reversed-phase Hypersil BDS C_8 column (4.6 × 200 mm, 5 μm, Agilent Co.) with a gradient elution. Eluent A was 50% acetonitrile containing 30 mmol L^{-1} ammonium formate (pH 3.7); B was 100% acetonitrile. The flow rate was constant at 1.0 mL min^{-1} and the column temperature was set at 30°C. The injection volume was 10 μL. The fluorescence excitation and emission wavelengths were set at λ_{ex} 260 nm and λ_{em} 430 nm, respectively. Gradient conditions: initial = 50% A + 50% B; 15 min = 30% A + 70% B (kept for 10 min); 25 min = 15% A + 85% B (kept for 3 min); 35 min = 100% B (kept for 5 min). MS conditions: Ion source type, atmospheric pressure chemical ionization (APCI source); nebulizer pressure 60 psi; dry gas temperature, 350°C; dry gas flow, 5.0 L min^{-1}; APCI Vap temperature, 350°C; capillary voltage, 3500 V; corona current, 4000 nA (pos).

Extraction of Fatty Acids From *Lycium chinese mill, Long pepper,* and *Evodiae fructus*

Lycium chinese mill (0.2 g), *Long pepper* (0.2 g), and *Evodiae fructus* (0.2 g) were separately triturated, and then put into three 50-mL volumetric flasks, respectively, (each containing 10 mL chloroform). The samples were extracted three times (20 min for each extraction) and then were allowed to incubate at room temperature for 24 hr. The contents were filtered, and the residues were washed with another 5 mL of chloroform; the combined chloroform was added to 1.5 mL of pyridine and was sonicated for 30 s. Then, the chloroform was evaporated to dryness with a stream of nitrogen gas. The residue was re-dissolved in 600 μL of DMF, and stored at 4°C until HPLC analysis.

Preparation of Standard Solutions

PPMB solution (5.0×10^{-2} mol L^{-1}) was prepared by dissolving 267.5 mg of PPMB in 10 mL DMF. Corresponding low concentration of labeling reagent solution (5.0×10^{-3} mol L^{-1}) was obtained by diluting the stock solution with DMF. The standard fatty acids for HPLC analysis at individual concentration of 1.0×10^{-4} mol L^{-1} were prepared by diluting the corresponding stock solutions (1.0×10^{-2} mol L^{-1}) with acetonitrile. When not in use, all solutions were stored at 4°C in a refrigerator.

Derivatization

The derivatization of PPMB with fatty acids was carried out in DMF solvent in the presence of K_2CO_3 catalyst (Figure 1). To a 2-mL vial 125 μL DMF, 4 mg K_2CO_3, 50 μL fatty acids (1.0×10^{-4} mol L^{-1} each) and 75 μL derivatization reagent (5.0×10^{-3} mol L^{-1}) were successively added. The vial was then sealed and heated at 95°C for 40 min in a thermostatic water-bath with shaking at 5-min intervals. After the reaction was completed, the mixture was cooled to room temperature. The 50 μL of this derivatization solution was diluted with 450 μL acetonitrile. The diluted solution (10 μL) was injected directly into the chromatograph.

RESULTS AND DISCUSSION

Fluorescence Excitation and Emission of PPMB and Its Derivatives

The excitation and emission spectra of PPMB and its derivatives were recorded by using the scanning mode of the fluorescence detector. The

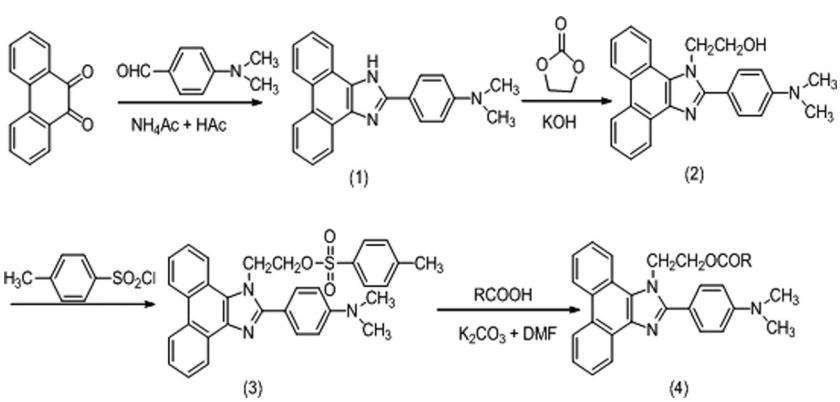

FIGURE 1 The synthesis of PPMB and the derivatization procedure for fatty acids.

excitation and emission spectra of PPMB in acetonitrile (1.0×10^{-4} mol/L) were shown in Figure 2A. The solution of the nonadecanoic acid derivative (C19-derivative, 2.0 μmol/L) in acetonitrile was used to obtain the fluorescence spectra (Figure 2B-2). As can be seen, the fluorescent excitation and emission intensity of PPMB itself showed a dramatically quenching relative to its derivative. This is probably due to the fact that the presence of sulfur atoms in PPMB molecular core structure resulted in a significant quenching in emission intensity. PPMB itself exhibited two excitation wavelengths at 260 nm and 360 nm, respectively (here, the intensity of the excitation wavelength at 360 nm relative to 260 nm was about twice). A relatively high emission wavelength of 430 nm was observed. However, PPMB derivatives exhibited intense fluorescence (FL) with an excitation maximum at λ_{ex} 260 nm and an emission maximum at λ_{em} 430 nm. It was found that the excitation wavelength at 360 nm had completely disappeared, a completely blue shift from 360 to 260 nm was observed for its derivatives. The selected spectrum at a monitoring wavelength of 260 nm as fluorescence excitation did not result in low sensitivity because the emission wavelength was kept essentially constant at 430 nm.

Comparison of Responses Between PPMB and TSPP for Fluorescence and MS

The pure PPMB-C19 and TSPP-C19 derivatives (each 10 mL, 1.0×10^{-4} M) was obtained by the semi-preparative HPLC separation. The fluorescence spectra were used to evaluate the fluorescence properties (Figure 2B). Recording spectra of PPMB-C19 and TSPP-C19 showed the same maximum excitation at λ_{ex} 258 nm in 100% acetonitrile. The molar absorption coefficients (ε) of PPMB-C19 in acetonitrile was 4.7×10^4 L

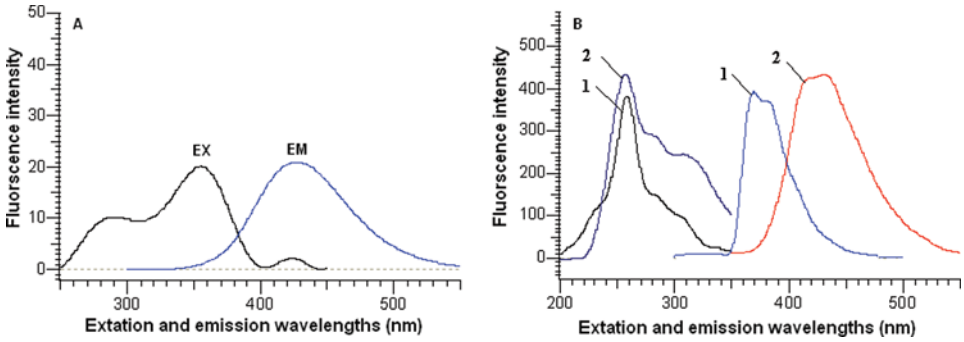

FIGURE 2 The fluorescence spectra of PPMB (A), TSPP-C19 derivative (B-1), and PPMB-C19 derivative (B-2). (Color figure available online.)

mol^{-1} cm^{-1} (TSPP-C19: λ_{259nm}, $\varepsilon = 6.0 \times 10^4$ L mol^{-1} cm^{-1}; λ_{320nm}, $\varepsilon = 1.8 \times 10^4$ L mol^{-1} cm^{-1}, UV spectra were not shown). The excitation intensity of PPMB-C19 in acetonitrile was 1.12% higher than that of TSPP-C19. The intensities of emission spectra of PPMB-C19 and TSPP-C19 were found to be similar. However, the wavelength of maximum emission of PPMB exhibited obvious red-shift and shown the maximum emission at λ_{em} 430 nm. It was probably due to the fact that the introduction of the N,N-dimethy function group (electron pair donor) into labeling reagent molecular core structure produced a large extent of p-π conjugation. In addition, the emission wavelengths in methanol and acetonitrile showed the similar results and exhibited no-solvent dependent.

In our previous studies, TSPP-fatty acid derivatives exhibited excellent fluorescence.[22] However, they also gave relatively low the ionization efficiency under APCI-MS in positive-ion mode as nitrogen atom in its core structure was intensively conjugated by benzene ring. This was great disadvantage to the post-column online sensitive MS identification for low contents of fatty acid compounds in real environmental samples. To provide high ionization efficiency coupled with excellent fluorescence, a water-soluble N,N-dimethylamino functional group was introduced into the core structure of PPMB molecule by its N-linked side chain to yield PPMB molecule. Thus, PPMB molecule bore a nitrogen-electronegative atom. It should enhance the ionization of PPMB-fatty acids significantly. MS ion current intensities for the derivatized fatty acids were compared to those obtained using TSPP as labeling reagent. The ratios for the MS ion current responses were as follows: $I_2/I_1 = 1.57 - 2.06$ (I_2 and I_1 are relative ion current intensities of PPMB- and TSPP-fatty acid derivatives, respectively, see Table 1). This should be attributed to the molecular structure of PPMB, in which its N-linked N,N-dimethylamino functional group contained a highly electronegative nitrogen atom and provided a site that could be easily accepted a $[H]^+$. Therefore, PPMB-fatty acid derivatives were not only sensitive to MS ionizable efficiency but also sensitive to fluorescence in the present structural forms. This character was of great importance to perform a sensitive fluorescence detection coupled with an online sensitive APCI-MS identification for the trace amounts of fatty acid compounds. In addition, the introduction of a water-soluble N,N-dimethylamino functional group into PPMB core structure could sufficiently improve the water-solubility of fatty acid-derivatives, and effectively reduced the retention time of PPMB- fatty acid derivatives for HPLC separation.

Stabilities of the Reagent PPMB and Its Fatty Acid Derivatives

When an anhydrous solution of PPMB itself in acetonitrile was heated at 95°C for 40 min, the derivatization yields for the fatty acids were not

TABLE 1 Comparison of Fluorenscence and MS Ion Current Responses

Fatty Acids	Fluorenscence Intensity			MS Intensity		
	H_1 (TSPP)	H_2 (PPMB)	H_2/H_1	I_1 (TSPP) ($\times 10^7$)	I_2 (PPMB) ($\times 10^7$)	I_2/I_1
C_{11}	205.31	202.01	0.98	1.33	2.63	1.98
C_{12}	189.09	192.03	1.02	1.55	2.94	1.90
C_{13}	197.79	200.65	1.01	1.92	3.34	1.74
C_{14}	180.17	171.15	0.95	1.80	3.20	1.78
$C_{18:2}$	177.80	167.77	0.94	2.10	3.57	1.70
C_{15}	142.50	142.01	1.00	1.70	3.16	1.86
C_{16}	193.39	188.66	0.97	1.95	3.67	1.88
$C_{18:1}$	354.78	376.34	1.06	3.80	7.61	2.00
C_{17}	187.89	204.80	1.09	2.40	4.68	1.95
C_{18}	201.34	185.65	0.92	3.00	5.04	1.68
$C_{20:1}$	242.05	271.56	1.12	5.00	8.90	1.78
C_{19}	165.42	147.53	0.89	2.65	4.16	1.57
C_{20}	159.12	195.72	1.23	3.00	6.09	2.03
$C_{22:1}$	212.90	235.64	1.11	4.80	9.89	2.06
$C_{24:1}$	287.62	265.78	0.92	5.25	8.87	1.69

obviously different. When PPMB itself in blank derivatization solution (with no analytes) was heated at 95°C for 40 min, a small amount of PPMB resulted in partial decomposition (<10%), but derivatization yields for the fatty acids were not obviously changed. This was probably due to the fact that the corresponding derivatives were stable and enabled further HPLC analysis for at least two weeks with normalized peak areas <0.43%. During the reaction, partial of the reagent decomposed at 95°C for 40 min, but the corresponding derivatives were stable and enabled further HPLC analysis for at least two weeks with no degradation when stored under refrigeration at 4°C.

Optimization for Derivatization

The effect of temperature on derivatization reaction was investigated from 45 to 95°C. The detection responses showed a remarkable increase in the temperature range and the constant fluorescence intensities for all fatty acids were obtained at 95°C. The reaction times from 15 to 60 min were investigated. The response increased with the increasing of reaction time until 40 min. As a result, constant fluorescence intensities for all fatty acids were obtained at 95°C for 40 min with the addition of five-fold excess of molar reagent to total molar fatty acids. No significant increase in derivatization yield was observed with the increase of derivatization time. Several types of reaction co-solvents were tested for the derivatization, including *N,N*-dimethylformamide (DMF),

acetonitrile, acetone, dichloromethane, ethyl acetate, dimethyl-sulfoxide (DMSO), and chloroform. The results indicated that DMSO and DMF gave the best results as assessed by the detector responses. A slight decrease in detector responses in acetonitrile solvents (ca. 60.2% response relative to that of DMF) was observed. Acetone, ethyl acetate, dichloroformethane, and chloroform gave the lowest responses (2.5%–28.9%) under the condition proposed. Although DMSO and DMF as reaction cosolvents gave the best and similar results for the derivatization, however, when DMSO was used as reaction cosolvent, several interfering peaks were observed for the chromatographic separation. In general, DMF was used as reaction co-solvent throughout this reaction. Several catalysts were tested in the study for derivatization, including NaAc, K_2CO_3, $K_2C_2O_4$, potassium citrate, and potassium tartrate. The results indicated that K_2CO_3 was the best basic catalyst and gave the highest detection response. The effect of amount of K_2CO_3 added on the derivatization yield was investigated. The results indicated that the maximum peak intensities could be achieved when 4 mg of K_2CO_3 was added (pH = 10.89). Detector responses did not increase significantly with further increasing amount of K_2CO_3.

Chromatographic Separation

To get better resolution for separation of fatty acid derivatives, several chromatographic columns, such as Spherisorb C18 (200 mm × 4.6 mm, 5 μm), Hypersil BDS C8 column (4.6 × 200 mm, 5 μm), Hypersil C18 (200 mm × 4.6 mm, 5 μm) and Akasil-C18 column (4.6 × 250 mm, 5 μm) were evaluated for the optimal chromatographic separation. Results indicated that the Hypersil BDS C8 column gave the most efficient separation at 30°C of column temperature. Thus for the simultaneous separation of fatty acid derivatives, a Hypersil BDS C_8 column was used and eluted with (A) 50% acetonitrile containing 30 mmol/L formic acid/ammonia buffer ($pH^* = 3.81$) and (B) 100% acetonitrile. The gradient elution from (A) to (B) was used to give the best separation with the shortest retention times and the sharpest peaks. As can be seen from Figure 3, all saturated and unsaturated fatty acid derivatives were separated with a good baseline resolution.

MS Identification

The ionization and fragmentation of the isolated fatty acid derivatives were studied under APCI-MS in positive-ion detection mode. As expected, PPMB fatty acid derivatives produced an intense molecular ion peaks at m/z

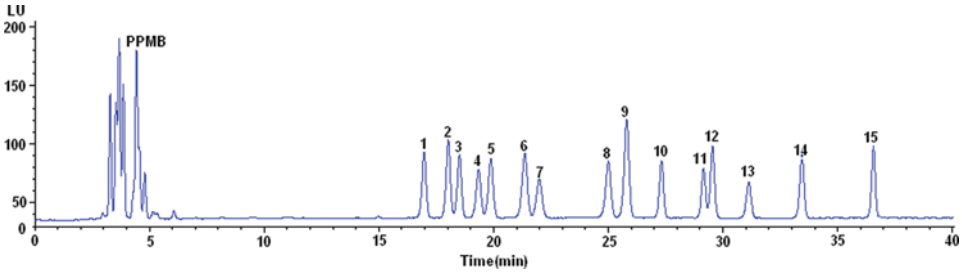

FIGURE 3 Chromatogram of a mixture of fatty acid standards. Chromatographic conditions: Column temperature at 30°C; excitation wavelength at λ_{ex} 260 nm, emission wavelength at λ_{em} 430 nm; flow rate = 1.0 mL min^{-1}; Peaks: 1, C20:5 (5, 8, 11, 14, 17-eicosapentaenoic acid); 2, C18:3 (8,11,14-octadecatrienoic acid); 3, C22:6 (2,5,8,11,14,17-docosahexenoic acid); 4, C14 (tetradecanoic acid); 5, C20:4 (6,9,12,15-arachidonic acid); 6, C18:2 (9,12-octadecadienoic acid); 7, C15 (pentadecanoic acid); 8, C16 (hexadecanoic acid); 9, C18:1 (9-octadecenoic acid); 10, C17 (heptadecanoic acid); 11, C18 (octadecanoic acid); 12, C20:1 (11-eicosenoic acid); 13, C19 (nonadecanoic acid); 14, C22:1 (12-docosenoic acid); 15, C24:1 (20-tetracosenoic acid). (Color figure available online.)

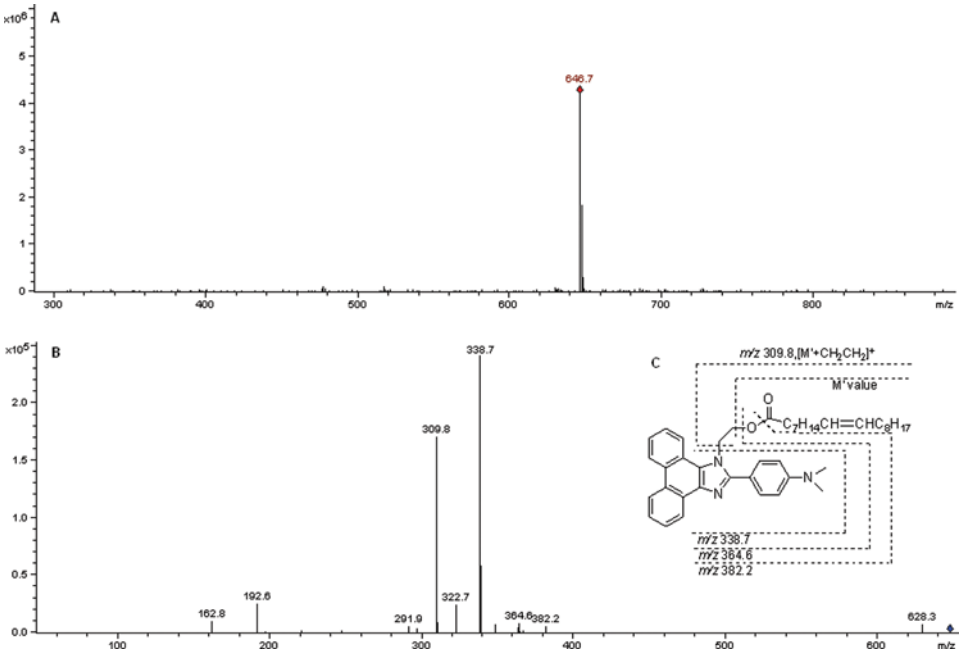

FIGURE 4 The profile of molecular ion chromatogram and scanning of the isolated representative n-C18:1 acid derivative (PPMB-C18:1). (A) Typical LC-MS profile of n-C18:1 acid derivative (PPMB-C18:1) from full scanning range from 100 to 800 amu with APCI in positive-ion detection mode. (B) Typical APCI-MS-MS profile of n-C18:1 acid derivative (PPMB-C18:1) from full scanning range from 100 to 600 amu with APCI in positive-ion detection mode. (C) The MS-MS cleavage mode of PPMB-C18:1 derivative. (Color figure available online.)

TABLE 2 MS and MS-MS Analysis

Fatty Acids	Fatty Acid Molecular Weight	Molecular Weight of Derivatives	Molecular Ion $[M+H]^+$	MS-MS Data	Specific Ions of $[M' + CH_2CH_2]^+$ and $[M\text{-}H_2O]^+$
C20:5	302	665	666.5	329.4, 338.7, 364.5, 382.7, 648.4 $[MH.H_2O]^+$	329.4, 648.4
C18:3	278	641	642.6	305.4, 338.7, 364.7, 382.8, 623.5 $[MH.H_2O]^+$	305.4, 623.5
C22:6	328	691	692.6	355.2, 338.7, 364.6, 382.9, 674.3 $[MH.H_2O]^+$	355.2, 674.3
C14	228	591	592.7	255.7, 338.7, 364.5, 382.5	255.7
C20:4	304	667	668.6	331.6, 338.7, 364.9, 382.8, 650.0 $[MH.H_2O]^+$	331.6, 650.0
C18:2	280	643	644.7	307.7, 338.7, 364.5, 382.2, 626.3 $[MH.H_2O]^+$	307.7, 626.3
C15	242	605	606.7	269.9, 338.7, 364.7, 382.6	269.9
C16	256	619	620.6	283.8, 338.7, 364.1, 383.0	283.8
C18:1	282	645	646.7	309.8, 338.7, 364.9, 382.2, 628.3 $[MH.H_2O]^+$	309.8, 628.3
C17	270	633	634.7	297.1, 338.7, 364.7, 382.6	297.1
C18	284	647	648.8	311.7, 338.7,364.9,382.5	311.7
C20:1	310	673	674.7	337.6, 338.7, 364.5, 382.6, 656.3 $[MH.H_2O]^+$	337.6, 656.3
C19	298	661	662.7	325.7, 338.7,364.8, 382.7	325.7
C22:1	338	701	702.6	365.8, 338.7, 364.9, 382.5, 684.4 $[MH.H_2O]^+$	365.8, 684.4
C24:1	366	729	730.6	393.7, 338.7, 364.5, 382.6, 712.4 $[MH.H_2O]^+$	393.7, 712.4

TABLE 3 Linear Regression Equations, Correlation Coefficients, Detection Limits, Repeatability for Peak Area, and Retention Time ($n=6$) and Recovery

Fatty Acids	Regression Equation	Correlation Coefficient	Detection Limit (fmol)	RSD of Retention time (%)	RSD of Peak Area (%)	Recovery (%)
$C_{20:5}$	$Y=35.27X+6.87$	0.9993	46.47	0.075	0.87	95.4
$C_{18:3}$	$Y=39.39X+6.39$	0.9996	39.72	0.094	1.10	89.2
$C_{22:6}$	$Y=32.60X+5.58$	0.9999	43.66	0.083	1.34	96.5
C_{14}	$Y=30.66X+4.79$	0.9998	44.58	0.068	1.17	95.7
$C_{20:4}$	$Y=19.86X+5.28$	0.9995	45.52	0.080	0.96	99.6
$C_{18:2}$	$Y=37.78X+8.16$	0.9993	53.33	0.077	0.71	100.5
C_{15}	$Y=29.62X+6.23$	0.9995	54.56	0.073	0.90	94.8
C_{16}	$Y=42.51X+9.56$	0.9995	32.09	0.049	0.64	104.7
$C_{18:1}$	$Y=57.79X+13.19$	0.9996	29.88	0.053	0.58	102.3
C_{17}	$Y=35.30X+8.90$	0.9995	32.55	0.056	0.85	98.5
C_{18}	$Y=23.40X+4.44$	0.9997	48.51	0.074	1.54	94.8
$C_{20:1}$	$Y=31.41X+4.10$	0.9998	41.23	0.066	1.73	88.3
C_{19}	$Y=28.05X+4.59$	0.9997	62.37	0.097	1.05	94.1
$C_{22:1}$	$Y=33.91X+5.73$	0.9995	37.71	0.082	0.93	92.5
$C_{24:1}$	$Y=32.90X+7.23$	0.9995	34.07	0.091	1.09	89.3

TABLE 4 Comparison of Detection Limit Between PPMB and Other Reported Reagents for Fatty Acids

Reagents	Methods	Wavelength (nm)	Detection Limit (fmol/10 μL)	Reference
PPMB	HPLC	260/430	29.88–69.37	This work
DAABD-AE	HPLC	430/570	110–660	[29]
MePZBD-AE	HPLC	430/570	90–900	[30]
ABD-PZ	HPLC	440/580	50–250	[31]
DBD-PZ-NH_2	HPLC	421/564	~227.5	[32]
HEC	HPLC	335/360	50–70	[17]
TSPP	HPLC	260/380	26.19–76.67	[22]
4-AF	CE		30–1500	[33]

$[MH]^+$. The collision-induced dissociation spectra of m/z $[MH]^+$ produced the specific fragment ions at m/z 338.7, m/z 364.6, m/z 382.2, and m/z $[M' + CH_2CH_2]^+$(M': fatty acid molecular moiety), respectively. The specific fragment ion m/z 338.7 was the corresponding protonated molecular core structure moiety (C-N bond cleavage). The characteristic fragment ion at m/z 364.5 came from the cleavage of C–O bond in the chain of $RNCH_2CH_2$–OCO (RN: core structure) and the fragment ion at m/z 382.7 came from the cleavage of O–CO ester bond in the chain of $RNCH_2CH_2O$–CO. The specific fragment ions at m/z $[M' + CH_2CH_2]^+$ was the corresponding protonated fatty acid ethyl ester moiety. Compared to saturated fatty acid derivatives, the MS-MS analysis of unsaturated fatty acid derivatives exhibited obvious difference. The collision-induced dissociation spectra of m/z $[M + H]^+$ usually contained the specific fragment ion at m/z $[MH\text{-}H_2O]^+$ that was produced by the loss of H_2O molecule. The selected reaction monitoring, based on the m/z $[M + H]^+ \rightarrow m/z$ $[MH\text{-}H_2O]^+$, m/z 382.2, m/z 364.6, and m/z 338.7 transitions, was specific for unsaturated fatty acid derivatives. The MS-MS analysis and corresponding cleavage mode for the representative PPMB-C18:1 derivative are shown in Figure 4A–C. MS and MS/MS data for all the fatty acid derivatives are shown in Table 2.

Linearity and Detection Limits for Derivatized Fatty Acids

Based on the optimum derivatization conditions, the linearity of the procedure was evaluated in the concentration range of 16.67 pmol-130 fmol by fluorescence detection. The linear regression equations, correlation coefficients, and detection limits for all fatty acid derivatives are listed in Table 3. All the fatty acid derivatives were found to provide excellent linear responses over this range with correlation coefficients >0.9993. The detection limits of each fatty acid (at a signal-to-noise ratio

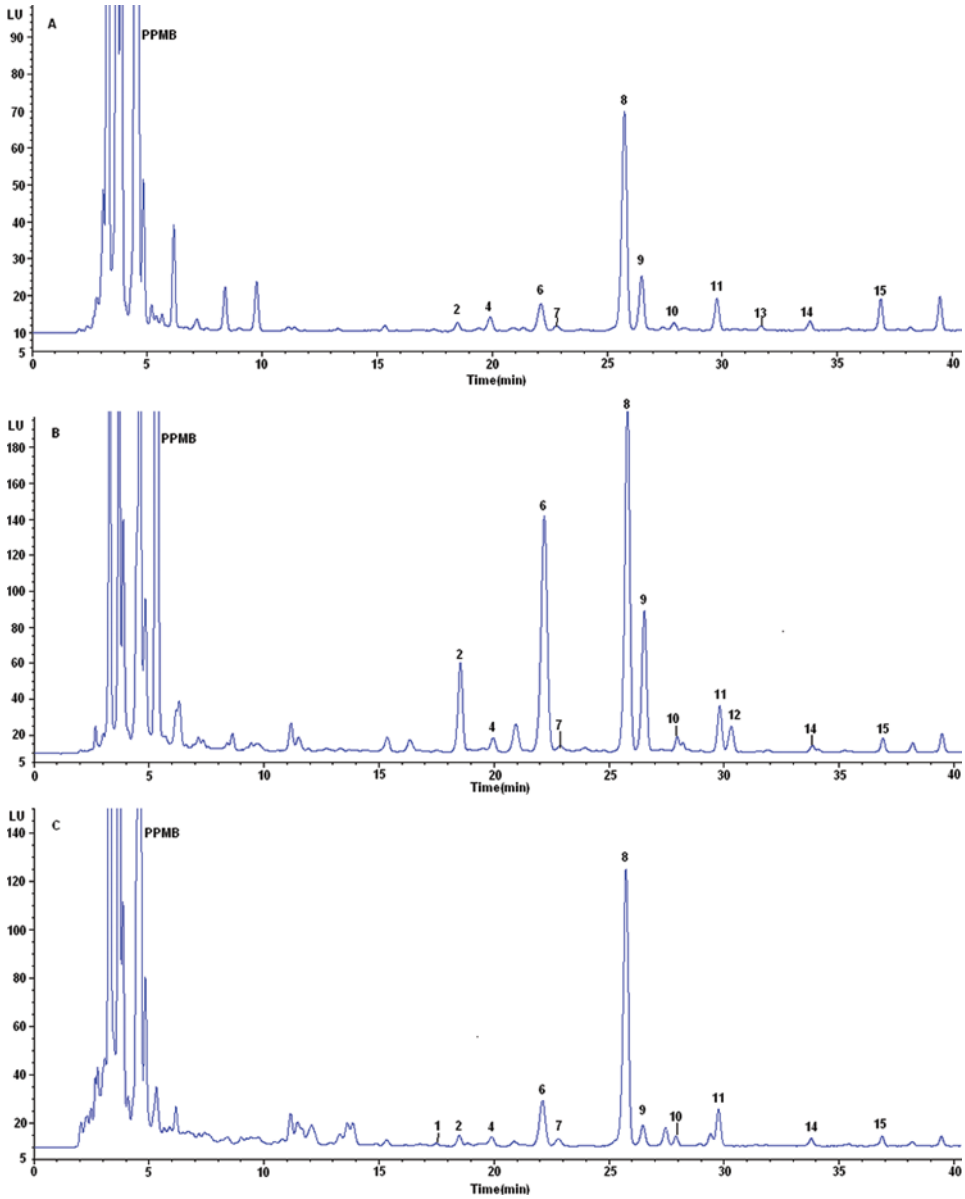

FIGURE 5 Chromatograms of fatty acids from the extracted samples of *Lycium chinense mill* (A), *Long pepper* (B), and *Evodiae fructus* (C). Chromatographic conditions and peaks as Figure 3. (Color figure available online.)

of 3:1) were from 29.88 to 62.37 fmol. This detection limits using PPMB as labeling reagent are compared with DAABD-AE,[29] MePZBD-AE,[30] ABD-PZ,[31] DBD-PZ-NH_2,[32] HEC,[17] TSPP,[22] and 4-AF,[33] respectively (Table 4). As it shown, the detection limit obtained with PPMB is better

than those reagents. In addition, PPMB showed wider wavelength than others. Thus, the comparison showed the advantages of the present method.

Analytical Repeatability, Precision, and Accuracy

The method repeatability was investigated by injecting 16.67 pmol fatty acid derivatives (10 μL injection, $n = 6$). The relative standard deviations (RSDs) of the retention times and peak areas varied from 0.0494% to 0.97% and from 0.5831% to 1.73%, respectively. The RSDs of the retention times and peak areas are shown in Table 3. Precision and accuracy: six replicates ($n = 6$) at 1.04, 4.17, and 16.67 pmol of fatty acids were used to make the low- to high-range concentrations. The mean intra- and inter-day precision for fluorescence detection were <3.62% and 5.18%, respectively. The accuracy for all fatty acids levels were in the range of 91.4%–105.1%. These indicated the satisfactory precision and accuracy of the developed method.

Analysis of Samples and Recovery

The chromatograms for the analysis of free fatty acids extracted from *Lycium chinese mill, Long pepper,* and *Evodiae fructus* samples are shown in

TABLE 5 Results of Fatty Acids from Real Samples

	Lycium Chinense Mill		Long Pepper		*Evodiae fructus*	
Components	Contents (μg/g)	RSD (%) ($n = 6$)	Contents (μg/g)	RSD (%) ($n = 6$)	Contents (μg/g)	RSD (%) ($n = 6$)
$C_{20:5}$	ND		ND		2.43	4.36
$C_{18:3}$	1.66	3.56	35.46	2.22	4.14	4.20
$C_{22:6}$	ND		ND		ND	
C_{14}	3.33	3.49	7.79	4.21	4.86	3.79
$C_{20:4}$	ND		ND		ND	
$C_{18:2}$	7.49	2.23	125.46	1.80	20.06	2.43
C_{15}	0.94	3.82	6.08	4.38	4.49	3.88
C_{16}	41.82	1.74	125.20	1.65	82.83	1.68
$C_{18:1}$	7.79	2.06	39.07	2.04	5.36	2.99
C_{17}	1.71	4.47	6.14	4.71	3.67	3.32
C_{18}	11.75	2.55	30.26	3.26	19.60	3.44
$C_{20:1}$	ND		15.67	3.14	ND	
C_{19}	1.16	4.35	ND		ND	
$C_{22:1}$	3.32	3.73	6.08	3.67	4.24	3.60
$C_{24:1}$	8.89	2.66	6.08	3.03	8.98	3.11

ND: not detectable or with values below LODs.

Figure 5. As can be seen, the saturated and unsaturated fatty acids were separated with a good baseline resolution, and the compositional data for the fatty acids are shown in Table 5. Amounts of saturated fatty acids with an even number of carbon atoms were clearly much higher than amounts of the adjacent saturated fatty acids with odd number of carbon atoms. The content of palmitic acid (C16) was obviously high relative to those of other fatty acids. In addition, it was found that three samples all contain five unsaturated fatty acids ($C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{22:1}$, and $C_{24:1}$), their contents might be dependent upon the type of samples.

The recoveries of 15 fatty acids were evaluated by the addition of a known amount of standard (10 μL, 1.0×10^{-4} mol L^{-1}, Table 3) into the pulverized *Long pepper* sample (*Lycium chinese mill* and *Evodiae fructus* were not evaluated). The extraction and derivatization methods were the same as described in the experimental section, and the analysis was carried out by three duplicates. The recoveries were 88.3%–104.7%.

CONCLUSIONS

A new fluorescence labeling reagent PPMB has been developed for the trace determination of fatty acids with HPLC separation and on-line MS identification. PPMB molecule contains N, N-dimethylamino functional group in its molecular core structure resulting in high ionization efficiency. The proposed method offers a number of advantages: (1) Derivatization of fatty acids used PPMB is relatively simple with little matrix interference; (2) Derivatives are sensitive to fluorescence and can be simultaneously obtained an online sensitive APCI-MS identification; and (3) Maximal yields close to 100% are observed with a 5- to 6-fold molar reagent excess. The proposed method has been successfully applied to the determination of fatty acids containing unsaturated fatty acids in real herbs samples with satisfactory results.

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